Anti-biofilms Activity of Peptides against *Burkholderia Thailandensis*

การศึกษากลไกของเปปไทด์ต้านจุลชีพในการยับยั้งไบโอฟิล์มจากแบคทีเรีย *Burkholderia thailandensis*

Pitawat Samutnavee (พิทวัส สมุทรนาวี) * Dr.Rina Patramanon (ดร. รินา ภัทรมานนท์)**

ABSTRACT

Burkholderia thailandensis is largely avirulent in humans and closely related to *Burkholderia pseudomallei,* which causes melioidosis. One of the virulent factors of gram-negative bacteria is the ability to produce biofilms. Both of *B. pseudomallei* and *B. thailandensis* have been reported to develop biofilms. Therefore, we interest to study meliodosis bacterial biofilms through *B.thailandensis* as model. Nowaday, Ceftazidem Ceftazidime (CAZ) is used antibiotic currently in melioidosis treatment from *B. pseudomallei* infection but increasing incidence of CAZ resistance has been reported. Antimicrobial peptides (AMPs) are alternative novel agents against drug-resistant pathogenic bacteria. Previous study showed that antimicrobial peptides have potential as anti-biofilm agents. In this study, we investigated the activity of CAZ and antimicrobial peptide CA-MA against *B.thailandensis* UE5. The results showed that 32 μg/mL of CAZ and could not eradicated the biomass of biofilms from *B. thailandensis* UE5 but can inhibit bacteria inside biofilms dramatically at 128 μg/mL. In contrast, CA-MA (Cecropin-Magainin) at 128 μg/mL can eradicate biomass of biofilms from *B. thailandensis* UE5 and can inhibit bacteria inside biofilms weakly with the same concentration. FTIR was used to examine and compare changes of the biofilms composition. We found that FTIR fingerprint of biofilms from *B.thailandensis* UE5 under control condition and CAZ treatment is not different. This indicated that CAZ did not alter biofilms composition. This study has demonstrated the different activity between antimicrobial peptide and antibiotic in inhibiting biofilms.

บทคัดย่อ

แบคทีเรียสายพันธุ์ *B.thailandensis* จัดว่าเป็นแบคทีเรียสายพันธุ์ที่ไม่ก่อโรคในมนุษย์ และมีความใกล้ชิดกับแบคทีเรียสายพันธุ์ *B.pseudomallei* ซึ่งเป็นสาเหตุก่อโรคเมลิออยด์ พบว่าเชื้อแบคทีเรียทั้งสองสายพันธุ์สามารถสร้างไบโอฟิล์ม ซึ่งเป็นปัจจัยหนึ่งในความรุนแรงของโรคเมลิออยด์ ในปัจจุบันยาปฏิชีวนะเซฟตาซิดีมถูกใช้เป็นยารักษาโรคเมลิออยด์ และพบว่าแบคทีเรียมีความทนทานต่อยาชนิดนี้มากขึ้น ในการรักษาโรคที่เกิดจากเชื้อแบคทีเรียที่มีความทนทานต่อยาปฏิชีวนะโดยใช้เปปไทด์ต้านจุลชีพ (antimicrobial peptides) พบว่าเปปไทด์ต้านจุลชีพมีประสิทธิภาพสูงในการทำลายไบโอฟิล์ม (anti-biofilms) ในงานวิจัยนี้ผู้วิจัยต้องการตรวจสอบกิจกรรมของยาปฏิชีวนะเซฟตาซิดีมและเปปไทด์ต้านจุลชีพกับไบโอฟิล์มของแบคทีเรียสายพันธุ์ *B.thailandensis* UE5 พบว่า ยาปฏิชีวนะเซฟตาซิดีมที่ความเข้มข้น 512 ไมโครกรัมต่อมิลลิลิตรไม่สามารถทำลายไบโอฟิล์ม พบว่าความเข้มข้นที่ 128 ไมโครกรัมต่อมิลลิตรสามารถยับยั้งแบคทีเรียที่อยู่ภายใต้ไบโอฟิล์ม ในทางกลับกัน เปปไทด์ต้านจุลชีพ CA-MA ที่ความเข้มข้น 128 ไมโครกรัมต่อมิลลิลิตร สามารถทำลายไบโอฟิล์ม และยับยั้งเชื้อแบคทีเรียภายใต้ไบโอฟิล์ม จากนั้นใช้เทคนิค FTIR เพื่อตรวจสอบและเปรียบเทียบองค์ประกอบของไบโอฟิล์มที่เปลี่ยนแปลง จากการตรวจสอบ FTIR fingerprint ของไบโอฟิล์มจาก *B.thailandensis* UE5 พบว่าไบโอฟิล์มในกลุ่มควบคุมและกลุ่มทรีตเมนท์ด้วยยาปฏิชีวนะเซฟตาซิดีมมีค่าไม่แตกต่างกัน แสดงให้เห็นว่ายาปฏิชีวนะไม่มีผลต่อองค์ประกอบของไบโอฟิล์ม จากการศึกษาในครั้งนี้แสดงถึงความแตกต่างของกิจกรรมจากเปปไทด์ต้านจุลชีพและยาปฏิชีวะจากในการยับยั้งไบโอฟิล์ม

Key words: Anti-biofilms agents; Antimicrobial peptides; *Burkholderia thailandensis*

คำสำคัญ: สารต้านไบโอฟิล์ม เปปไทด์ต้านจุลชีพ แบคทีเรีย *Burkholderia thailandensis*

**Student, Master of Science Program in Biochemistry, Department of Biochemistry, Faculty of Science, Khon Kaen University*

*** Assistant Professor, Department of Biochemistry, Faculty of Science, Khon Kaen University*

Introduction

Burkholderia pseudomallei is a Gram-negative, soil saprophyte and the causative agent of melioidosis, an endemic disease throughout southeast Asia and northern Australia (West et al., 2008) the Indian subcontinent, Iran, and Central and South America (Kanthawong et al., 2010; Tyson et al., 2009) *B. pseudomallei* is intrinsically resistant to many antimicrobial agents. CAZ is the drug of choice that mostly effective for melioidosis treatment (Sawasdidoln et al., 2010). Up to a few years ago, *B. thailandensis* was considered to be an avirulent biotype of *B. pseudomallei*, as the two organisms are very similar to one another in most characteristics. *B. thailandensis* has similar environment distribution, indistinguishable morphology, share similarity genome and majority of virulence factors similar to *B. pseudomallei* (Yoon et al., 2010), excepting for virulence factors against man and animals (Kespichayawattana et al., 2004) *B.thailandensis* is useful as model organism which less virulent than *B.pseudomallei*, suit for researcher to study pathogenicity of meliodosis (West et al., 2008) and does not require a biosafety level 3 containment facility to study *B.thailandensis* . *thailandensis* has similar environment distribution, indistinguishable morphology, share similarity genome and majority of virulence factors similar to *B. pseudomallei* (Yoon et al., 2010), excepting for virulence factors against man and animals (Kespichayawattana et al., 2004) *B.thailandensis* is useful as model organism but is less virulent than *B.pseudomallei*, suiting for researcher to study pathogenicity of meliodosis (West et al., 2008). Moreover, one does not require a biosafety level 3 containment facility to study *B.thailandensis*. One of the virulent factors of Gram-negative bacteria is the ability to produce biofilms. Both of *B. pseudomallei* and *B. thailandensis* has been reported to develop biofilm (Taweechaisupapong et al., 2005; Andreae et al., 2014). Biofilms are complex, localized and fixed networks of microbial cells enclosed in a self-produced polymeric matrix. (Jorge et al., 2012) Growing biofilms are resistant to several antibiotics or other antimicrobial agents up to 1,000 times than planktonic cells (Sawasdidoln et al., 2010; Park et al., 2011) which cause problem with medical device. Research has been focused in biofilms prevention and two main steps in destroying biofilms. Those are 1) dispersion or eradication of the biofilm exopolysaccaride (EPS) and 2) penetration into bacteria embedded in the EPS (Park et al., 2011; Jorge et al., 2012). Typically, lethal or inhibiting concentrations of antibiotics need to increase when treating bacteria with biofilms because they are might unable to translocate into EPS to reach the bacterial cells inside. In contrast, antimicrobial peptides are believed to have potential as anti-biofilm due to their different activity (Park et al., 2011). We perform anti-biofilm activity of CAZ and antimicrobial peptide CA-MA against *B.thailandensis* UE5 biofilm.

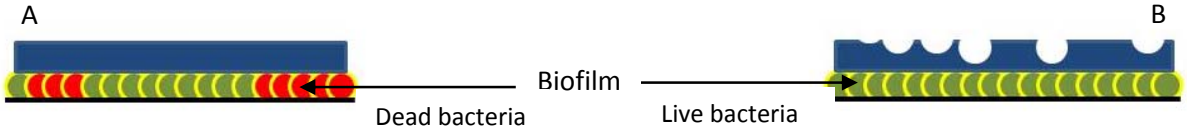

Figure 1 Biofilm reduction can be achieved in two ways, 1) reduction of surface-bound population called penetration mechanism (A) and removal of the established biofilm called dispersion or eradication mechanism (B)

Methods

Biomass of biofilms quatification

The quantitative biomass assay for biofilm was performed according to method described by Taweechaisupapong et al. (2005) with briefly modification. The BHI was inoculated with 2% inoculum (v/v) of *B.thailandensis* UE5 from overnight fresh culture and incubated further for 18 h. The 18-h culture was adjusted to give an optical density (OD) at 540 nm of 1.0. 150 µL of adjusted suspension was added into the well of the sterile 96-well plate. The plates were incubates aerobically at 37°C for 24 h. After that, the supernatant fluid of each well was aspirated to remove nonadherant cells and the wells were washed with 150 µL of sterilized PBS. Then, CAZ was added into biofilm- forming wells at range of concentrations (4,096, 2,048, 1,024, 512 and 256 µg/mL) , for CA-MA the range of concentrations are 512, 256, 128, 64 and 32 µg/mL. Then, the wells were further incubated for 24 h, each well was washed with sterilized PBS, and fixed with 200 µL of absolute methanol for 15 min and dried at room temperature. Each well was stained for 5 min with 150 µL of Hucker crystal violet. Excess stain was removed with running tap water. The plates were air dried and the dye bound to biofilm was solubilized with 150 µL of 33% (v/v) glacial acetic. The OD of each well was measured at 630 nm using microplate reader (SpectraMax M5). The biomass of biofilm was determined twice and the results reported were the average±S.D. from these two independent experiments and report as OD at 630 nm.

Quantification of bacterial viability embeded biofilm

Bacterial viability were determined by resazurin staining technique to predict biofilm penetration by antimicrobial agents (Siala et al., 2014; Chung et al., 2014) with briefly modification from Skogman, et al. (2012) and Taweechaisupapong et al (2005). The BHI was inoculated with 2% inoculum (v/v) of *B.thailandensis* UE5 from overnight culture and incubated further for 18 h. The 18-h culture were adjusted to give an optical density (OD) at 540 nm of 1. 150 µL of adjusted suspension was added into sterile 96-well plate. The plates were incubated aerobically at 37°C for 24 h. After 24 h, the supernatant fluid of each well was aspirated to remove nonadherant cells then, wells were washed with 150 µL of sterilized PBS. Both CAZ and CA-MA were added into biofilm-forming wells at range of concentration (512, 256, 128, 64 and 32 µg/mL), and further incubated for 24 h. After that, wash wells with sterilized PBS. Each well was stained with 20 µM of resazurin with 150 µL each well for 4 h. Then, the resazurin left in the wells were measured by fluorescent mode with excitation wavelength and emission wavelength at 540 nm and 590 nm, respectively. The bacterial viability was determined twice and the results reported were the average±S.D. from two independent experiments and report as florescent intensity.

FTIR analysis

To determine effect of antimicrobial agents with *B. thailandensis* UE5 biological compositions by using FTIR analysis technique, described by Ansari et al. (2013) and Taweechaisupapong et al (2005) with briefly modification. The BHI was inoculated with 2% inoculum (v/v) of *B.thailandensis* UE5 from overnight culture and incubated further for 18 h. The 18-h culture was adjusted to give an optical density (OD) at 540 nm of 1.0, then added 4 mL of adjusted culture into sterilized glass plate. Plates were

incubated at 37°C, 60 RPM of agitation. Then, 4 mL of each concentration of antimicrobial agents were added into biofilm-forming glass plates 512 µg/mL, further incubation for 24 h. Then, wash plates with sterilized PBS as above and dried. Treated and untreated biofilms were analyzed with FTIR spectrometer (**Bruker TENSOR27**. Bipfilms materials were powdered. Transfered biofilm powdered to ATR crystal, and then transmission spectra were recorded from 4000 cm-1 with 4 cm-1 spectral resolution. The biofilm composition was determined twice and result reported as FTIR spectra (cm^{-1}).

Results

Quantification of biofilms biomass

In this experiment, antimicrobial agents were added onto the established biofilms to observe the effect of antimicrobial agents in dispersing through established biofilms. The results showed that biofilm of *B. thailandensis* UE5 treated with CA-MA at 64 µg/mL was slightly and significantly eradicated than control group. Noticed that, CA-MA at 32 µg/mL has no effect with biofilms (Fig 2). Similarly, the established biofilms treated with CAZ at 32 µg/mL and higher concentration cannot eradicated significantly *B.thailandensis* biofilms (Fig 3).

Quantification of bacterial viability embeded biofilms

In this experiment CAZ and CA-MA were applied onto the established biofilms of *B. thailandensis* UE5 to observe the penetration

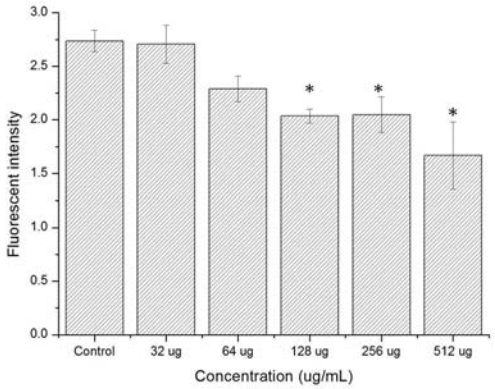

Figure 2 Biomass of *B. thailandensis* UE5 biofilm treated with CA-MA at 32-512 µg/mL. Three wells are used for each CA-MA. Data are shown as mean±S.D. of values from two independent experiments. *p<0.05 vs control

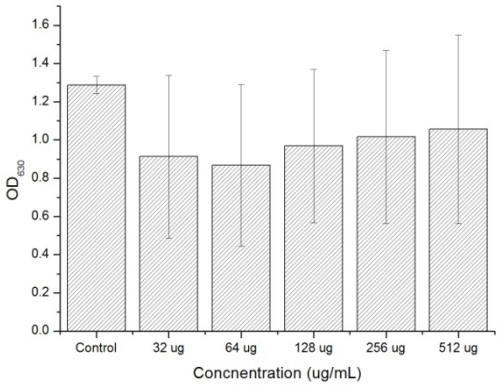

Figure 3 Biomass of *B. thailandensis* UE5 biofilm treated with CAZ at 32-512 µg/mL. Three wells are used for each CAZ. Data are shown as mean±S.D. of values from two independent experiments.*p<0.05 vs control

activity of both antimicrobial agents into biofilm. The results showed that CA-MA at 128 µg/mL concentration have effect to inhibit bacteria inside biofilm. (Fig 4) On the other hand, CAZ at 32 µg/mL significantly inhibited cell viability but CAZ at higher concentration (128 µg/mL) can dramatically and significantly inhibited the cell viability embeded biofilm (Fig 5)

FTIR analysis

The FTIR spectra exhibited well defined spectra regions that correspond to the vibration of

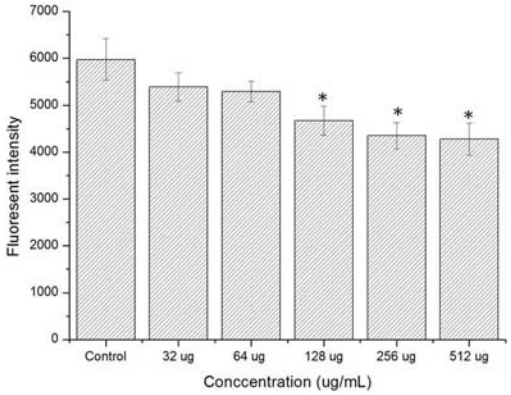

Figure 4 Bacterial viability quantification of *B. thailandensis* UE5 treated with CA-MA at 32-512 µg/mL. Three wells are used for each CA-MA. Data are shown as mean±S.D. of values from two independent experiments. *p<0.05 vs control

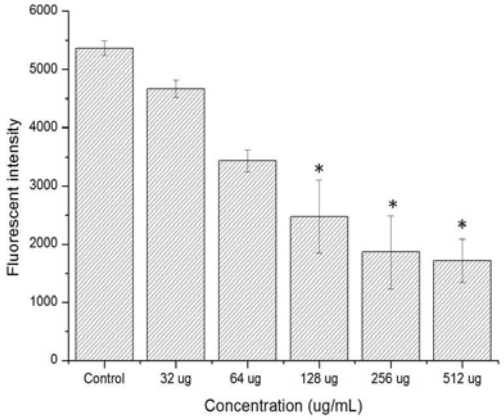

Figure 5 Bacterial viability quantification of *B. thailandensis* UE5 treated with CAZ at 32-512 µg/mL. Three wells are used for each CAZ. Data are shown as mean±S.D. of values from two independent experiments. *p<0.05 vs control

chemical group from *B. thailandensis* UE5 biofilm (Fig6) . A tentative assignment of bands corresponding to functional groups is summarized in Table 1. The biofilm in the control group showed the peak at 3278 cm^{-1}, indicating the N-H and O-H stretching vibration found in polysaccharides and proteins. The peak at 2956 cm^{-1} indicated the C-H symmetric stretch of CH_2 functional groups dominated by fatty acid chains. The peak at 2872 might represented C-H symmetric stretch of CH_2 in fatty acids. The peak at 1641 cm^{-1} might represent amide I, stretching C=O in amide. The peak at 1543 cm^{-1} represented amide II. The peak at 1454 cm^{-1} represented C-H bending from CH_2 and CH_3. The peak at 1399 cm^{-1} might represented C=O stretching for deprotonated COO^- group. The peak at 1233 cm^{-1}might represent P=O symmetric stretching in phosphodiester fucntional group of DNA/RNA polysaccharide backbone. The peak at this range 1156 and 1072 cm^{-1} represented oligo and polysaccharides vibration. The peak at 859 cm^{-1} represent glycosidic linkage with anomeric region (Wang et al., 2013; Schmitt et al., 1995) . When comparing the control with the CAZ-treated group, the peak at 2956 cm^{-1} disappeared and the peak at 1645 cm^{-1} exhibited shrinkage as shown in Fig 6.

Discussion and conclusion

In this study, we treated the established biofilm in the 96-well plate with the antibiotic, CAZ and the antimicrobial peptide, CA-MA in order to follow biofilm biomass. The result shown that CA-MA at 128 µg/mL have effect on *B.* **Table 1**

Assignment of functional groups associated in FTIR spectra of biofilms

FTIR frequency (cm^{-1})	Definition of the spectral assignment
3290	N-H and O-H stretching vibration: polysaccharides and proteins
2930	C-H symmetric stretch of CH_2 functional groups dominated by fatty acid chains
2850	C-H symmetric stretch of CH_2 in fatty acids
1647	Amide I, stretching C=O in amides; C-N, -NH and $–NH_2$ bending of protein and peptides amide
1548	Amide II, N-H bending and C-N stretching in amides; bending, -NH and NH_2 of amides
1539	Amide II, N-H binding, C-N stretch of proteins and peptides
1453	C-H bending from CH_2 and CH_3
1402	C=O symmetric stretching for deprotonated COO^- group and C-O bending from COO^-
1243	P=O symmetric stretching in phosphodiesters, monoester phosphate functional groups, functional group of DNA/RNA polysaccharide backbone structure and phosphorus-containing carbohydrates
1114	Polysaccharide region vibration
1084	C-OH, C-O, C-C stretching and C-O-C, C-O ring
1056	Vibration of carbohydrates (oligo and polysaccharides); C-O-P, P-O-P and P=O stretching of polyphosphate products
916	C-O, C-C stretching, C-OH, C-O-C deformation of carbohydrates
858	Glycosidic linkage type “anomeric region”

Table 1

Assignment of functional groups associated

Applied from Wang et al. (2013) and Schmitt et al.(1995)

thailandensis UE5 biofilm. In contrast, CA-MA at 32 µg/mL show no effect with *B. thailandensis* UE5, i.e. This result are similar which CA-MA can inhibit *P.aeuginosa* biofilm (Dosler, Karaaslan, 2014) whom related with Burkholderia spp. (Fig. 2). CAZ at 512 µg/mL have no effect to reduce biomass (Fig 3). The result suggested that the composition of EPS from biofilms could prevent CAZ destroy biofilm by eDNA form EPS which attach and degrade CAZ. Noted that our concentrations were all lower than minimum biofilm eradication concentration (MBEC) of antimicrobial agents against the biofilm of *P. aeruginosa* ATCC 27853 (Dosler, Karaaslan, 2014). We could conclude that antimicrobial agents exhibited eradication effect toward *B. thailandensis* UE5 biofilm which antibiotics cannot.

Next, we followed bacterial viability to predict penetration activity of the antimicrobial agents. The result showed that CA-MA at 128 µg/mL have effect on bacterial viability, meaning CA-MA could penetrate the biofilms to inhibit growth of bacterial cells attached inside (Fig 4). On the other hand, CAZ at lowest concentration (32 µg/mL) slightly inhibit the bacteria viability which could be the effect of β-lactamase from *B.thailandensis* that can inhibit B-lactam ring from CAZ (Poole, 2011) and the higher the concentration (128 µg/mL and further), the higher inhibition. (Fig 5). This suggested that CAZ which is active against only dividing cells was not very efficient at eradicating the biomass of *B. thailandensis* UE5 biofilms (Chung, Toh, 2012) Therefore, CAZ is effective for penetrating the biomass to inhibit the the bacterial viability. This will confirm by study which have shown that the penetration of antibiotics not limited in bacterial biofilm (Bordi, Bentzman, 2011) . Finally, we performed FTIR analysis of CAZ untreated and treated biofilm. ATR-FTIR absorpion requires a change of molecular vibration of polar group. These peaks represent functional group vibration in the main biomolecular composition such as lipids, proteins, polysaccharides, phospholipids, nucleic acids, and other carbohydrates. The result showed that *B. thailandensis* UE5 biofilm was composed of polysaccharides and protein and others. The FTIR spectra at wavenumber 2956 and 1641 cm^{-1} disappeared when treated with CAZ at 512 µg/mL, indicating that CAZ altered the amide component such as *N*-acetylmuramic acid (NAM) , and *N*-acetylglucosamine (NAG) which contain amino

group. CAZ also altered the cell membrane of bacteria containing acyl group. FTIR-analysis also showed that CAZ inhibited peptidoglycans from cell membrane.

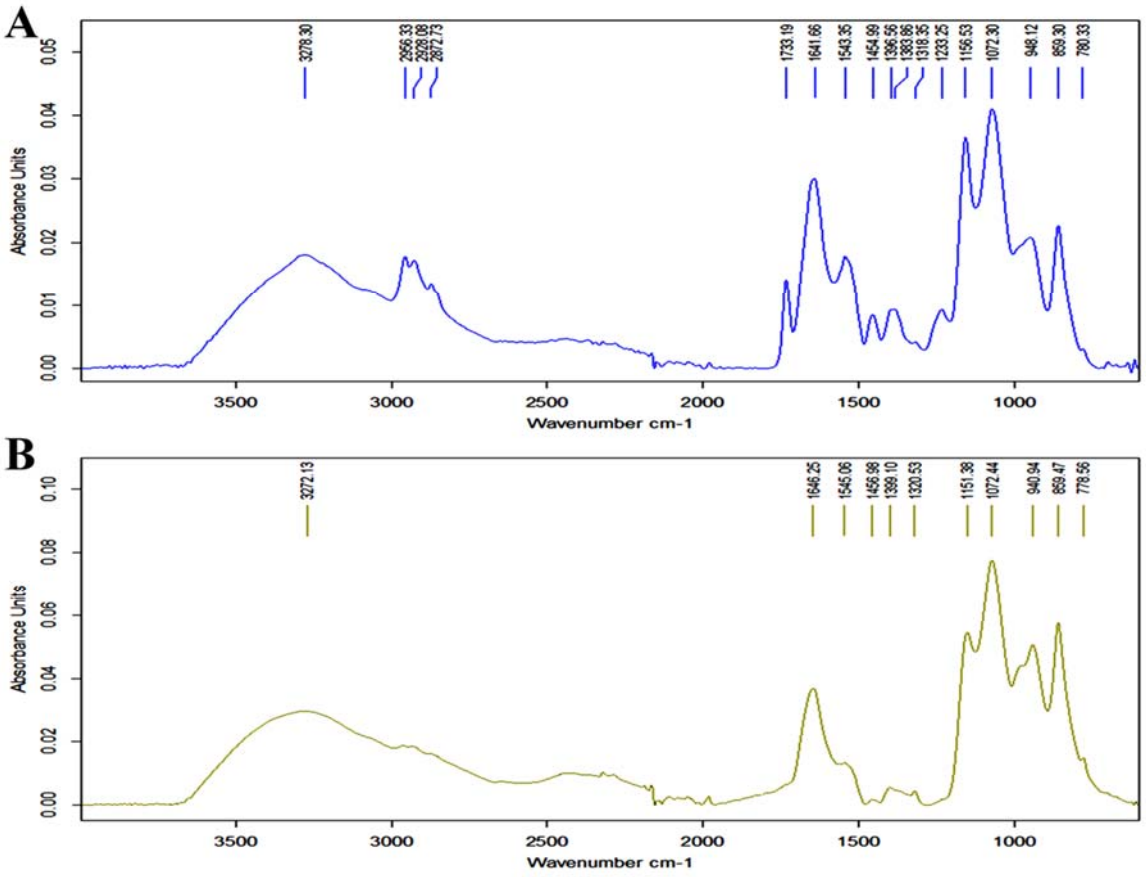

Figure 6 FTIR spectra of *B. thailandensis* UE5 biofilms. (A) the spectra of untreated biofilm after 48 h. (B) the spectra of biofilm treated with 512 µg/mL of CAZ after 48 h

Our finding demonstrate that CA-MA have slight effect with biofilms but CAZ, which have no effect. Due to low concentration or these agents have no activity with biofilms. These result confirmed by FTIR. For penetration study shows that, CA-MA have slightly bacterial inhibition effect but CAZ have dramatically bacterial inhibit effect with *B.thailandensis* biofilms. Due to CAZ might translocated through EPS, then inhibit bacteria embeded inside biofilms.

Acknowledgements

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